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Research article

Effects of everolimus on circadian gene expression and cell fate in synchronized Caco-2 cells

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ABSTRACT

Objectives: Circadian rhythms regulate key biological processes, including cell proliferation and metabolism, and their disruption is implicated in colorectal cancer (CRC). mTOR signaling interacts bidirectionally with the circadian clock, yet how mTOR inhibition modulates clock gene dynamics and cellular behavior in intestinal models remains unclear. This study aimed to investigate the effects of everolimus, an mTOR inhibitor, on circadian gene expression, cell viability, apoptosis, and cell cycle progression in synchronized Caco-2 cells, with consideration of cell confluency and Circadian Time (CT).

Methods: Caco-2 cells were synchronized using serum shock at 20 % and 70 % confluency. Time-series samples were collected across multiple CTs (CT6–CT60). Gene expression (*BMAL1, PER2, mTOR*) was assessed by qRT-PCR using *ACTB* and *RPLP0* as reference genes. Rhythmicity was evaluated via Cosinor analysis. Cell viability, apoptosis, and cell cycle dynamics were analyzed using the Muse™ Cell Analyzer following everolimus treatment (1–50 μM).

Results: RPLP0 proved to be a more stable reference gene than *ACTB*. *BMAL1* exhibited stronger rhythmic expression than *PER2*, particularly at 20 % confluency. Everolimus (50 μM) significantly reduced cell viability in a time-dependent manner, with the greatest effect at CT6 and CT18 ($p < 0.0001$). Apoptosis was markedly increased at CT6 (+38.5 %) and moderate at CT18, indicating circadian modulation of drug sensitivity. Serum shock alone shifted cell cycle distribution, decreasing G0/G1 and increasing G2/M phase populations ($p < 0.01$). Everolimus altered *BMAL1* and *PER2* expression rhythms and significantly reduced mTOR expression at CT30, where baseline mTOR levels were highest. Cosinor analysis confirmed rhythmicity in *BMAL1/RPLP0* and *mTOR/RPLP0* profiles under low confluency.

Conclusion: Our findings demonstrate that everolimus influences circadian gene expression and exerts time-dependent antiproliferative and pro-apoptotic effects in Caco-2 cells. These results support the potential of circadian timing as a strategy to enhance mTOR-targeted therapies in CRC.

1. Introduction

The circadian timing system governs various molecular, physiological, and behavioral processes via 24-h rhythms. The central clock in the suprachiasmatic nucleus (SCN) of the hypothalamus regulates this system, synchronizing it with the light–dark cycle and coordinating self-oscillating peripheral clocks across the body [1]. In vitro, peripheral clocks in cultured cells can sustain rhythmicity; however, they lose synchronization in the absence of external cues. Agents such as serum, heat shock, dexamethasone, and forskolin are frequently employed to

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induce synchronization in cell culture models [2].

The gastrointestinal tract possesses strong circadian clocks that are especially responsive to feeding signals. The timing of food intake in the colon can surpass SCN input, underscoring the functional autonomy of intestinal clocks [3,4]. Disruption of circadian rhythms in the gut is associated with an elevated risk of colorectal cancer (CRC), as evidenced by studies involving genetically modified mice (e.g., Per2, Bmal1, Clock knockouts) and analyses of human CRC tissues exhibiting altered clock gene expression [5].

Caco-2 cells, derived from human colorectal adenocarcinoma, are extensively utilized as an in vitro model for the intestinal epithelium. The presence of circadian oscillations in these cells is noted; however, their heterogeneity and the attenuation of rhythms over time may restrict their translational applicability. Nonetheless, they serve as a valuable instrument for investigating circadian regulation within the intestinal framework [6–8].

Recent studies have revealed bidirectional interactions between the circadian clock and the mechanistic target of rapamycin (mTOR) pathway, a key regulator of cell growth, metabolism, and cancer progression [9]. mTOR activity modulates clock protein expression and oscillatory properties, while core clock components such as PER2 and REV-ERBα influence mTOR signaling [9,10]. In intestinal and CRC models, these interactions affect tumorigenesis, proliferation, and stem cell behavior [11–13]. Moreover, preclinical studies with the mTOR inhibitors suggest that its efficacy and toxicity are influenced by the time of administration [14–16].

Everolimus, a rapalog and clinically approved mTORC1 inhibitor, is widely used in oncology and transplant medicine. The mTOR signaling pathway plays a central role in cell growth, metabolism, and tumor progression, and everolimus has been applied as a targeted therapy in cancers such as pancreatic, renal, and breast malignancies. Preclinical data indicate that mTOR activity in tumors follows circadian rhythms and that aligning drug administration with these rhythms may enhance efficacy and reduce toxicity [14–16]. For example, everolimus administration at phases of heightened mTOR activity (e.g., ZT12) has been associated with tumor mass reduction and improved survival [14], whereas dosing at other circadian times altered toxicity profiles, including immunological responses [15,16]. Clinical evidence also suggests that treatment timing can modulate patient outcomes; in breast cancer, administration closer to the beginning of the activity span was linked to improved tolerability and potentially better survival [17]. In this context, evaluating how everolimus influences circadian gene expression and cell fate in an intestinal epithelial model provides critical insights into its potential role in colorectal cancer chronotherapy.

Despite growing evidence linking mTOR signaling with circadian regulation, the mechanisms by which mTOR inhibition alters clock gene rhythms and related cellular functions in the intestinal epithelium are still not well understood. This study investigates the time-of-day-

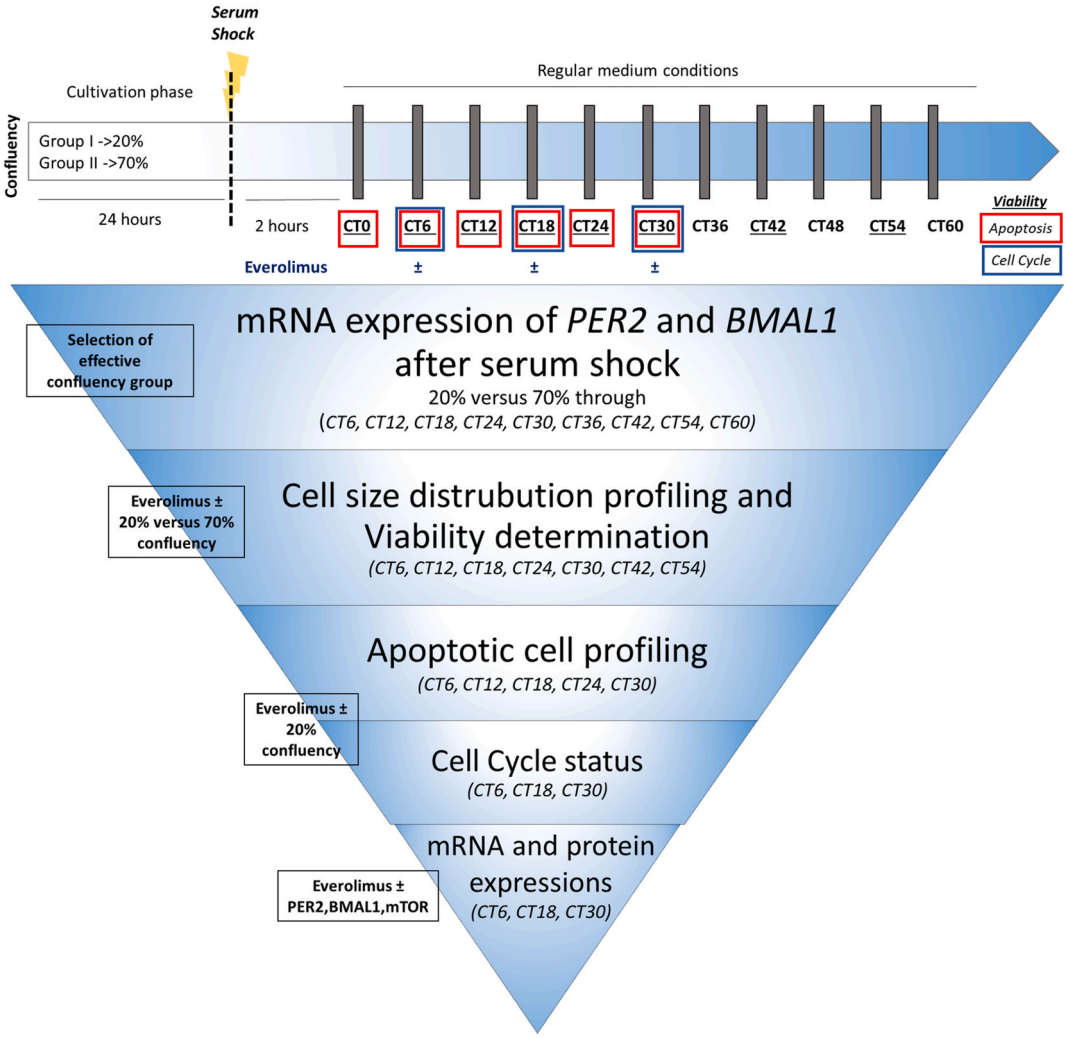

Fig. 1. Experimental Workflow and Circadian Time Allocation. Caco-2 cells were seeded at 20 % or 70 % confluency, followed by a 2-h serum shock and return to standard medium. Sampling occurred every 6 h up to CT60. The upper panel indicates CTs used for viability (underlined), apoptosis (red frame), and cell cycle/gene expression (blue frame). The lower panel summarizes the experimental flow. CT: Circadian Time.

dependent effects of everolimus in synchronized Caco-2 cells by [1]: characterizing *BMAL1* and *PER2* oscillations under varying confluency conditions [2]; evaluating the impact of everolimus on cell viability, apoptosis, and the cell cycle within a circadian framework; and [3] correlating these effects with alterations in *mTOR* transcript levels. The findings support the rationale for chronotherapy in colorectal cancer.

2. Materials and methods

An overview of the experimental setup, including confluency, serum shock, and time-point grouping, is shown in Fig. 1.

2.1. Cell culture and synchronization

Caco-2 cells (ATCC® HTB-37™, CVCL_0025), a human colorectal adenocarcinoma cell line, were cultured in DMEM/F12 (1:1) supplemented with 20 % FBS, 1 % penicillin-streptomycin, and 0.1 % primocin at 37 °C in 5 % CO_2. Experiments were conducted between passages 9–15. Cells were seeded at 3×10^4 (20 % confluency) or 1.5×10^5 cells/well (70 % confluency) in 24-well plates. 20 % and 70 % confluences were selected to represent proliferative and semi-confluent cellular states, respectively. Synchronization was achieved by 2-h serum shock using DMEM/F12 with 50 % FBS, followed by media replacement [18]. Samples were collected at indicated Circadian Times (CTs) for gene expression (CT6–CT60) and viability/cell count (CT6–CT54) analyses. CT0 corresponds to the time of serum shock initiation. All experiments were independently triplicated.

2.2. Everolimus treatment

Everolimus (ApexBio, USA) was dissolved in DMSO (10 mM stock) and applied at final concentrations of 1, 5, 10, and 50 μM. For apoptosis and cell viability studies, 50 μM was used at selected CTs post-synchronization. All experiments were conducted using a 24-h treatment period with everolimus.

2.3. Cell viability

Viability and live cell counts were measured using the Muse™ Cell Analyzer with Count & Viability Assay Kit (Merck Millipore, USA) at each CT. Trypsinized cells were stained according to manufacturer instructions, and viability was calculated as mean ± SEM from three independent experiments.

2.4. RNA isolation and qRT-PCR

Total RNA was extracted using EcoPURE RNA Kit (Ecotech, Turkiye), quantified via Multiskan GO (Thermo Fisher, USA), and equalized to 100 ng/μL cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). qRT-PCR was performed with SensiFAST SYBR® No-ROX Kit on a CFX Connect system (Bio-Rad). Primer sequences were designed using Primer3Plus [19]: Primer sequences for *PER2*; F: AAATCCGCTACCACCCCTTC, R: AAGGCAGCAAAGCTGACTCTC *BMAL1*; F: AAGGGAAGCTCACAGTCAGAT, R: GGACATTGCGTTGCATGTTGG, *ACTB*; F: CATGTACGTTGCTATCCAGGC, R: CTCCTTAATGTCACGCACGAT, and *RPLP0*; F: AGCCCAGAACACTGGTCTC, R: ACTCAGGATTTCAATGGTGCC. Relative gene expression was calculated via the $2^{-\Delta\Delta Ct}$ method [20].

2.5. Apoptosis and cell cycle analysis

Apoptosis was assessed using Muse™ Annexin V & Dead Cell Assay Kit at CT6, CT12, CT18, CT24, and CT30 with or without 50 μM everolimus. Cells were stained, incubated in the dark for 20 min, and analyzed using standard gating. Apoptosis was reported as % viable, early/late apoptotic, or dead cells. Cell cycle status (G0/G1, S, G2/M) was determined using Muse™ Cell Cycle Assay Kit at CT6, CT18, and CT30 under identical conditions.

2.6. Cosinor Analysis

To assess circadian rhythmicity, Cosinor Analysis (CA) was conducted using the CosinorPy Python package [21]. Rhythmicity parameters (MESOR, amplitude, acrophase) were derived from trigonometric regression models fitted to *PER2* and *BMAL1* expression data under varying confluency conditions (details and equations provided in **Supplementary Methods**).

2.7. Statistical analysis

Data were analyzed using one-way, two-way or three-way ANOVA with Tukey's post hoc test (GraphPad Prism 9.0). Results are presented as mean ± SD, with significance thresholds set at $p < 0.05$.

3. Results

3.1. RPLP0 is a more reliable reference gene than ACTB

PER2 and *BMAL1* expression levels normalized to *RPLP0* were consistently higher in amplitude and rhythmicity than those normalized to *ACTB*, in both 20 % and 70 % confluency groups (Fig. 2A and B, Table S1–S2). Cosinor fitting demonstrated clearer 24-h rhythmicity at 20 % confluency compared to 70 % (Fig. 2C and D). Circular phase plots further confirmed better 24 h fit for *BMAL1/RPLP0* at 20 % confluency (Fig. 2E vs. 2G), while a 48 h period fit was more appropriate at 70 % confluency (Fig. 2H). Among all, *BMAL1/RPLP0* showed the strongest rhythmicity, while *PER2* expression remained arrhythmic across periods tested (Tables 1 and 2).

3.2. Confluency and serum shock influence on cell viability

Cell viability varied significantly with confluency, serum shock, and CT ($p < 0.0001$). A significant interaction between these variables was observed via three-way ANOVA ($p = 0.0203$) (Fig. S1). Serum shock reduced viability in both confluency groups, but statistical significance at individual CTs was observed only at 20 % confluency, particularly at CT6 ($p < 0.05$).

3.3. Everolimus reduces viability in a dose- and time-dependent manner

Treatment with 50 μM everolimus significantly reduced viability across most CTs in both confluency groups (Fig. 3A and B). In 20 % confluency cells, the most prominent viability reduction occurred at CT12 and CT18 (e.g., $p < 0.0001$ for CT12), while in 70 % confluency cells, CT6 showed the lowest viability ($p < 0.0001$). Based on these findings, subsequent apoptosis and gene expression studies were focused on CT6, CT18, and CT30 under 20 % confluency.

3.4. Serum shock alone alters apoptotic profile

Serum-shocked cells showed decreased viability and increased apoptosis compared to untreated controls. Significant increases in late apoptosis were detected at CT18 and CT30 (Fig. 4A), suggesting synchronization alone can alter apoptotic dynamics.

3.5. Everolimus induces apoptosis most strongly at CT6

Everolimus (50 μM) induced the highest increase in total apoptosis at CT6 (+38.5 %, $p < 0.0001$), with moderate effects at CT18 and minimal at CT30 (Fig. 4B–C, Fig. S2). Time-dependent differences in viability and apoptosis support a chronopharmacological effect.

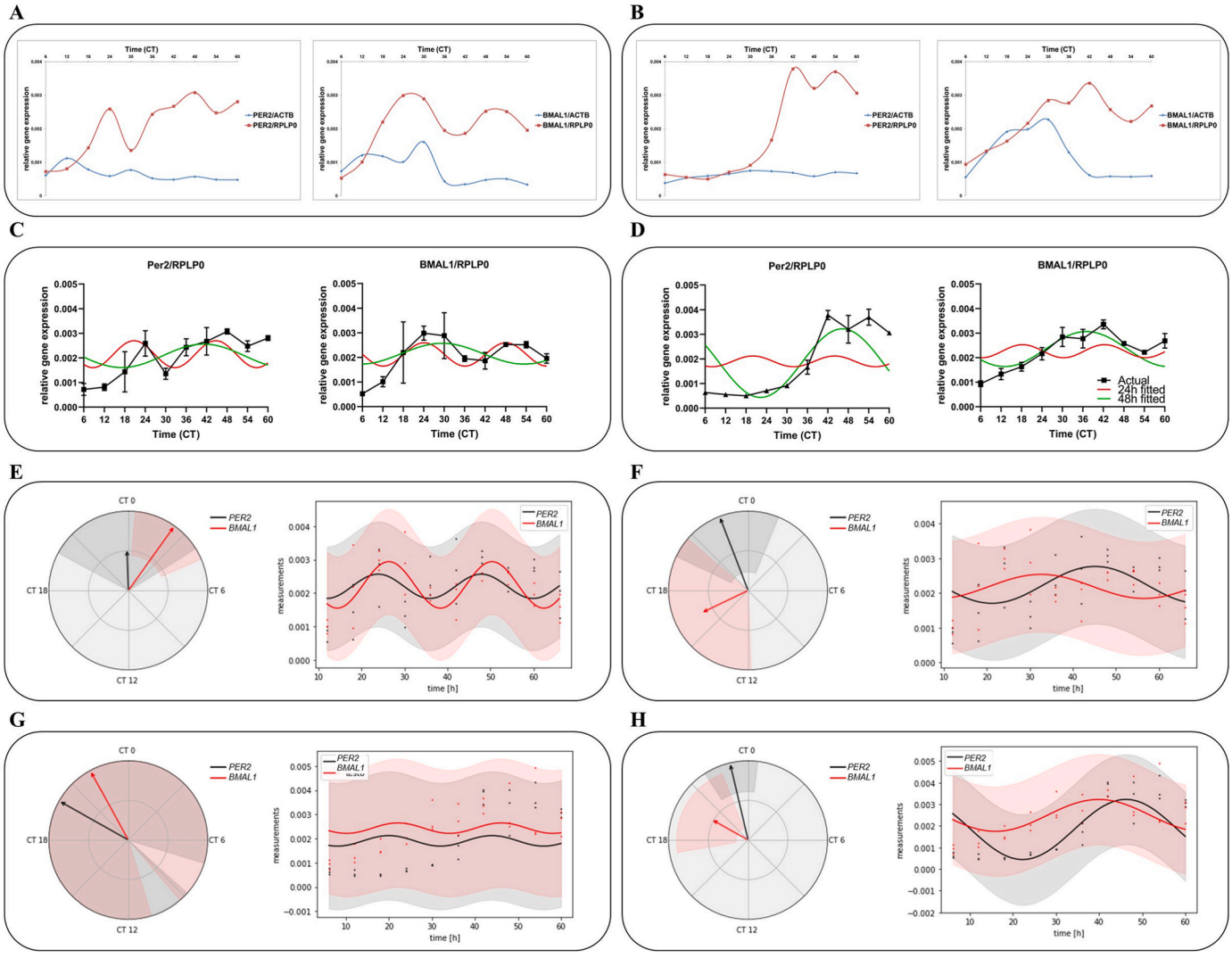

Fig. 2. Circadian Gene Expression Profiles of PER2 and BMAL1 Under Different Confluency and Reference Gene Conditions. Relative mRNA levels of PER2 and BMAL1 normalized to ACTB or RPLP0 at (A) 20 % and (B) 70 % confluency in serum-shocked Caco-2 cells. Cosinor-fitted expression curves (24 h and 48 h) for PER2/RPLP0 and BMAL1/RPLP0 (C) 20 % and (D) 70 % confluency in serum-shocked Caco-2 cells. Circular phase plots illustrating acrophase and rhythm fit for 24 h and 48 h periods (E) 20 % and (H) 70 % confluency in serum-shocked Caco-2 cells. BMAL1/RPLP0 showed stronger rhythmicity than PER2, especially at (G) 20 % confluency compared to (H) 70 % confluency in serum-shocked Caco-2 cells.

Table 1
Significance (p) values showing the rhythmicity of clock genes *PER2* and *BMAL1* relative to two housekeeping genes *ACTB* and *RPLP0* according to different periods in serum shocked Caco-2 cells at 20 % confluency.

Period (h)	*PER2/ACTB*	*PER2/RPLP0*	*BMAL1/ACTB*	*BMAL1/RPLP0*
18	0.141	0.567	**0.029**	0.579
24	0.988	0.174	0.580	0.061
30	0.724	0.149	0.770	**0.000**
36	0.886	0.543	0.348	**0.005**
42	0.587	0.781	**0.023**	**0.015**
48	0.174	0.178	**0.000**	**0.035**
54	0.055	**0.012**	**0.000**	**0.034**

Rhythms are considered significant when $p < 0.05$. Significant p values are shown in bold.

Table 2
Significance (p) values showing the rhythmicity of clock genes *PER2* and *BMAL1* relative to two housekeeping genes *ACTB* and *RPLP0* according to different periods in serum shocked Caco-2 cells at 70 % confluency.

Period (h)	*PER2/ACTB*	*PER2/RPLP0*	*BMAL1/ACTB*	*BMAL1/RPLP0*
18	0.893	0.726	0.374	0.978
24	0.684	0.849	0.496	0.488
30	0.066	0.657	0.210	0.700
36	0.091	0.645	**0.018**	0.083
42	0.270	0.092	**0.000**	**0.009**
48	0.615	**0.000**	**0.000**	**0.001**
54	0.692	**0.000**	**0.000**	**0.000**

Rhythms are considered significant when $p < 0.05$. Significant p values are shown in bold.

3.6. Serum shock drives cell cycle toward proliferation

Compared to untreated controls, serum-shocked cells showed a significant reduction in G0/G1 (−28.1 %, $p = 0.0006$) and increase in G2/M phase (+20.9 %, $p = 0.0014$), indicating acceleration of cell cycle progression (Fig. 5, Fig. S3).

3.7. Everolimus alters clock gene expression rhythms

Everolimus significantly modulated *BMAL1* and *PER2* expression

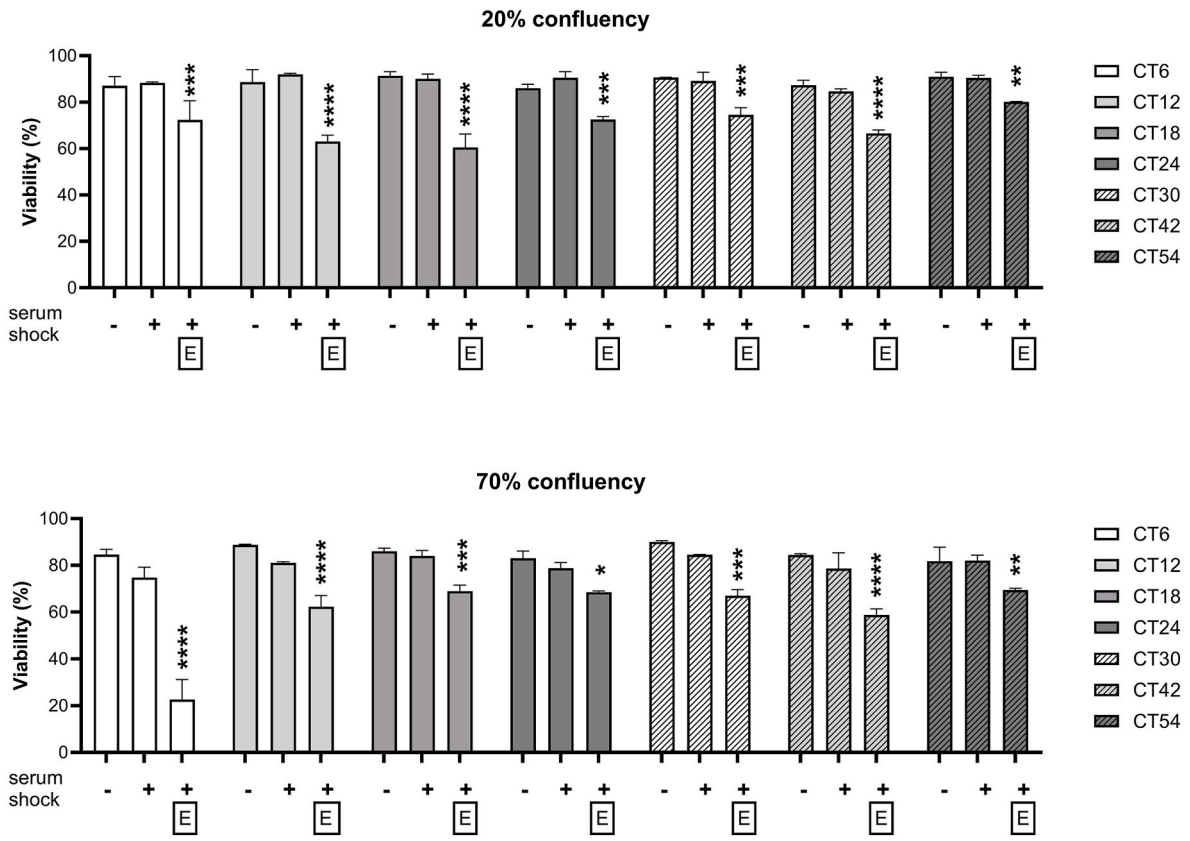

Fig. 3. Viability Analysis Following Synchronization and Everolimus Treatment. (A) At 20 % confluency, 50 μM everolimus significantly reduced viability at all CTs. **(B)** At 70 % confluency, viability also declined with 50 μM everolimus at all CTs. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. CT: Circadian Time. E: Everolimus.

relative to *RPLP0*, particularly decreasing *PER2* at ZC30 and increasing *BMAL1* at CT6 and CT18. Statistical significance was only detected in *RPLP0*-normalized data (Fig. 6, Table S1–S2).

3.8. *mTOR expression is time-regulated and suppressed by everolimus*

mTOR expression followed a rhythmic pattern, peaking at CT30 (*RPLP0* group) and CT18 (*ACTB* group). Everolimus suppressed *mTOR* expression in a time-dependent manner, with the most pronounced reduction at CT30 in *RPLP0*-normalized samples.

4. Discussion

This study investigated the circadian regulation of core clock genes and cellular responses to everolimus in synchronized Caco-2 cells. We examined the influence of serum-shock synchronization, confluency, and dosing time on gene expression (*BMAL1, PER2, mTOR*), cell viability, apoptosis, and cell cycle dynamics. Our findings suggest that both synchronization and dosing time critically shape cellular sensitivity to mTOR inhibition, with implications for chronotherapy in colorectal cancer.

Circadian disruption is a hallmark of many malignancies, including colorectal cancer (CRC) [22]. Numerous studies show that loss of *PER1* and *PER2* expression is consistently linked to with tumor progression and poor prognosis [23–26], whereas the impact of *BMAL1* is context-dependent—some cohorts report its up-regulation in tumour tissue and even an association with longer survival [23–26] or improved oxaliplatin response [27]. In our Caco-2 model, a 2-h serum shock successfully re-synchronized the clock and elicited rhythmic *BMAL1* and *PER2* transcription, albeit with lengthened periods and damped amplitudes relative to non-transformed epithelia. Interestingly, *BMAL1* oscillations appeared more regular than those of *PER2* in this setting, contrasting with several cancer-cell studies in which *PER2* rhythms are generally more robust while *BMAL1* amplitude and stability decline with increasing malignancy. This divergence suggests that the relative resilience of individual clock loops can vary according to cell type, malignant stage and synchronization protocol.

We found that *RPLP0* was a more reliable reference gene than *ACTB*, particularly under conditions of serum manipulation and low confluency. This aligns with earlier studies validating *RPLP0* stability in Caco-2 cells and other colon adenocarcinoma lines [28,29].

Confluency played a significant role in modulating synchronization and drug responsiveness. Lower confluency (20 %) supported more pronounced rhythmic expression and stronger time-dependent responses to everolimus. In contrast, high confluency (70 %) dampened these effects. These findings underscore the importance of cell density in circadian studies and reinforce the need for carefully controlled culture conditions.

Everolimus, a well-characterized mTORC1 inhibitor, demonstrated time-dependent effects on cell viability and gene expression. A substantial reduction in viability and increase in apoptosis were observed at CT6, with weaker effects at CT18 and minimal response at CT30. These results mirror studies in breast cancer models showing that everolimus efficacy varies by circadian time [30]. Moreover, our findings extend this concept by demonstrating a clear rhythmic component in apoptosis induction, suggesting optimal timing may enhance therapeutic benefit.

Everolimus also modulated *BMAL1* and *PER2* expression, decreasing *PER2* most prominently at CT30 and increasing *BMAL1* at CT6 and CT18. These effects may reflect bidirectional crosstalk between mTOR and the circadian clock, where mTOR activity influences the stability, translation, and post-translational modification of core clock proteins [31]. Conversely, clock components such as PER2 and REV-ERBα can

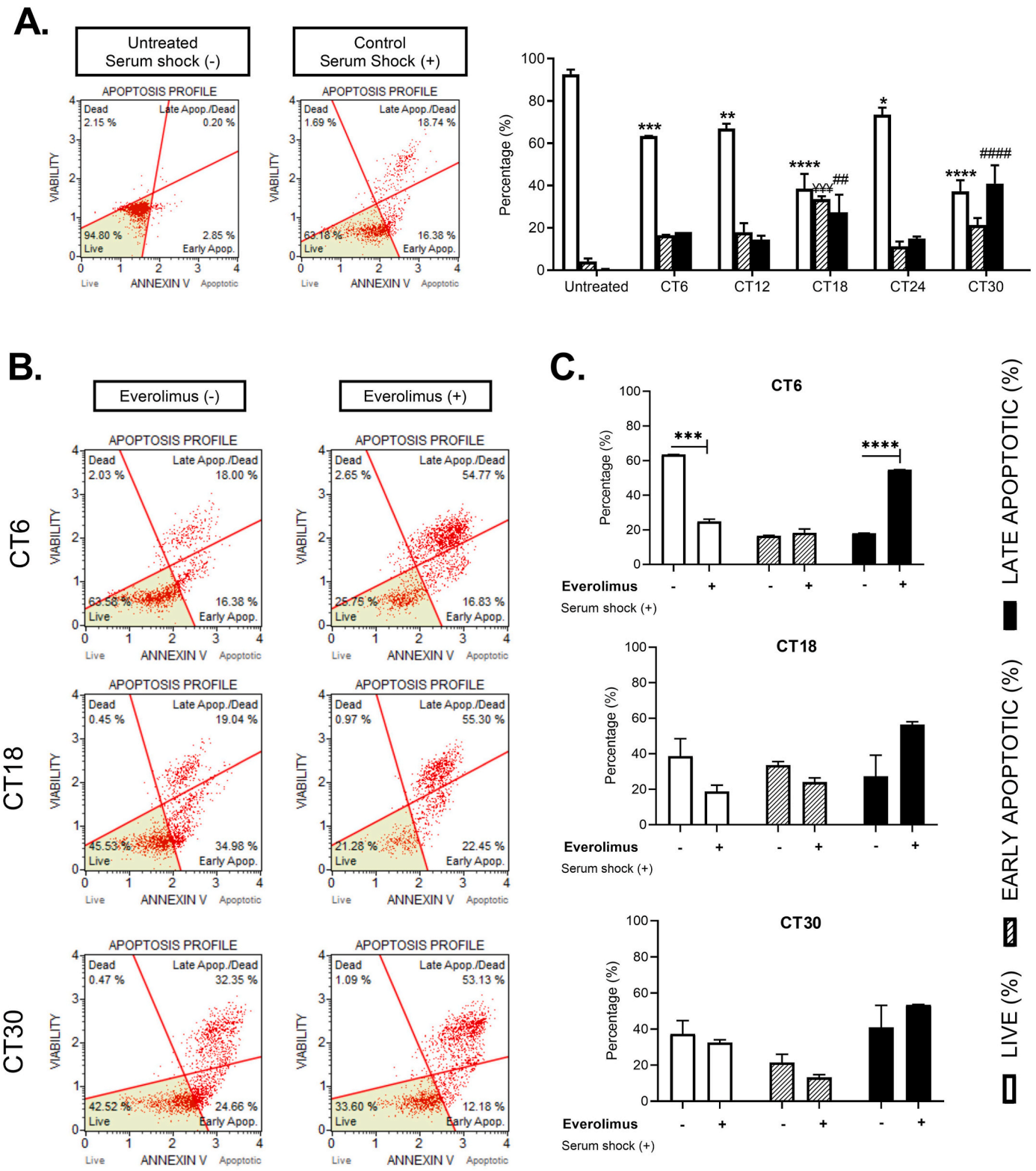

Fig. 4. Apoptotic Cell Profiles of Synchronized Caco-2 Cells Treated with Everolimus. (A) Serum-shocked cells showed reduced viability and increased apoptosis, especially at CT18 and CT30. Statistics are versus untreated controls: Live cells: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.Early apoptotic: ¥¥¥p < 0.001. Late apoptotic: ##p < 0.01, ####p < 0.0001. **(B–C)** Everolimus (50 μM) further increased total apoptosis, with the strongest effect at CT6. ***p < 0.001, ****p < 0.0001. CT: Circadian Time.

modulate mTORC1 activity via TSC1 signaling [10,32]. This feedback loop is increasingly recognized as critical in regulating metabolism, proliferation, and cancer progression. However, it is also important to consider that the disruption of *PER2* and *BMAL1* rhythms observed after everolimus treatment may not solely result from canonical mTOR inhibition. Everolimus has been reported to exert broader regulatory actions, including modulation of mitochondrial function and oxidative stress responses [33], alteration of autophagy dynamics [34,35] and

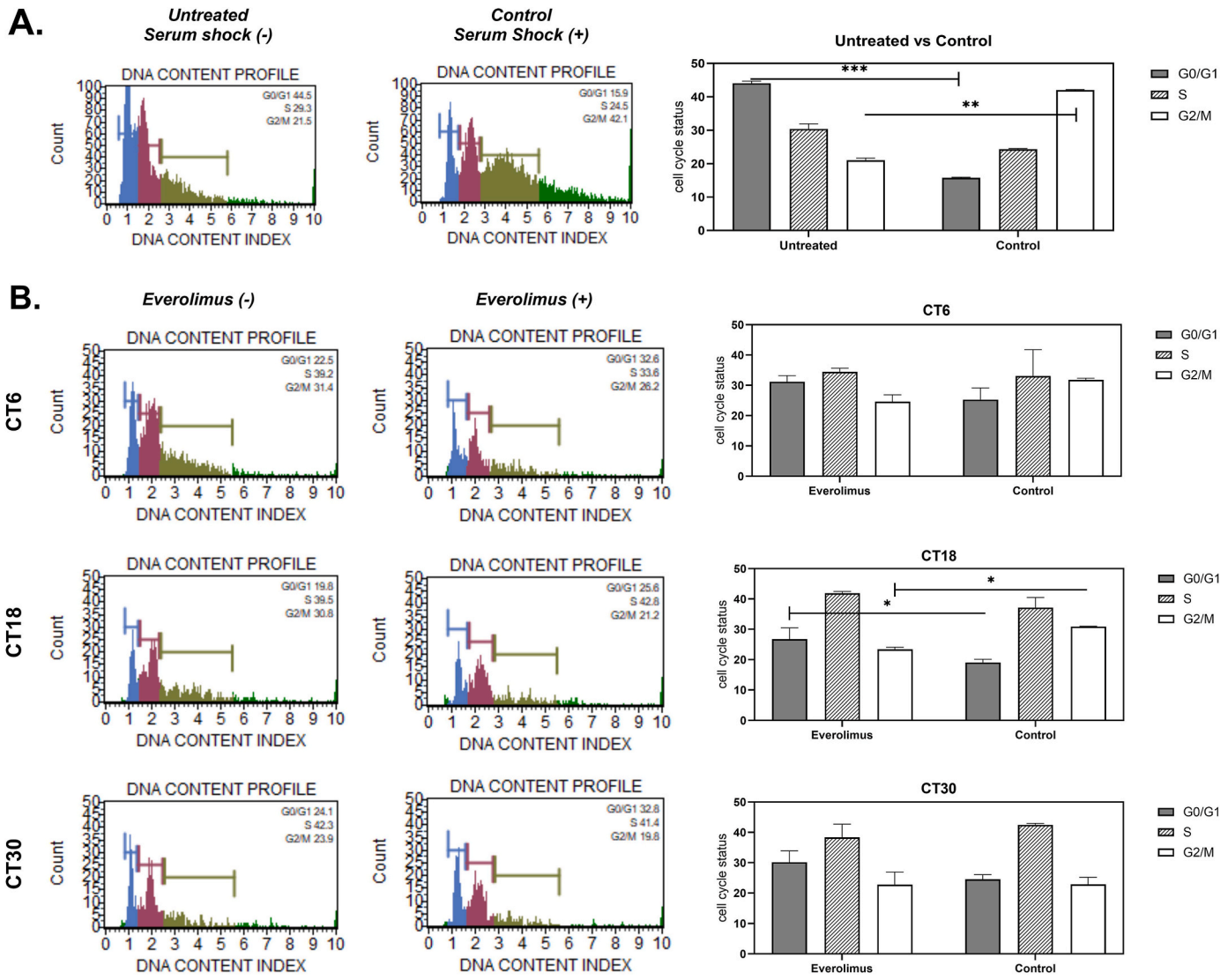

Fig. 5. Cell Cycle Distribution Altered by Serum Shock and Everolimus. (A) Serum shock reduced G0/G1 and increased G2/M phase populations in Caco-2 cells. **(B)** Everolimus (50 μM) induced G0/G1 arrest at CT18. *p < 0.05.

suppression of ABC transporters such as ABCB1 and ABCG2 in intestinal epithelial cells [36]. These pleiotropic effects could indirectly influence circadian gene expression networks, highlighting the need for cautious interpretation of our results and for future studies using complementary approaches (e.g., genetic mTOR silencing or comparison with other rapalogs) to disentangle direct versus indirect effects on the clock machinery.

In line with these molecular changes, *mTOR* transcript levels also displayed circadian rhythmicity, peaking at CT30. Everolimus treatment reduced *mTOR* expression in a time-dependent manner, with the greatest suppression occurring when basal expression was highest. These results further support the concept that mTOR inhibition is not uniform over time and may benefit from chronobiological optimization [15,16].

Finally, cell cycle analysis revealed that serum-shocked Caco-2 cells exhibited reduced G0/G1 and increased G2/M phase distribution, indicating accelerated cell cycle progression post-synchronization. Studies have shown that a 2-h 50 % FBS pulse delivers a potent mitogenic stimulus that activates MAPK/ERK and PI3K–Akt–mTOR signalling, up-regulates cyclin D1 and degrades p27ˆKip1, thereby shortening G0/G1 and synchronizing the population into S phase within 6–10 h [37–39]. Twenty-four hours later, the same cohort has completed DNA replication and accumulated in G2/M, explaining the reciprocal drop in G0/G1 and rise in G2/M fractions observed here. Everolimus treatment at CT6 reinforced G1 arrest and triggered apoptosis, suggesting synergism between synchronization and drug timing.

A limitation of this study is the use of a single cell line (Caco-2). Although Caco-2 cells are a well-established intestinal epithelial model exhibiting robust circadian and mTOR signaling characteristics, additional cell line validation will be necessary to generalize these findings.

5. Conclusion

Our results demonstrate that the timing of everolimus administration significantly alters its antiproliferative and pro-apoptotic effects in Caco-2 cells. These findings support a circadian dimension to mTOR-targeted therapies and highlight the potential of chronotherapy in optimizing CRC treatment strategies.

CRediT authorship contribution statement

Dilek Ozturk Civelek: Writing – review & editing, Methodology, Investigation, Funding acquisition, Conceptualization. **Beyza Goncu:** Writing – review & editing, Methodology, Investigation, Funding

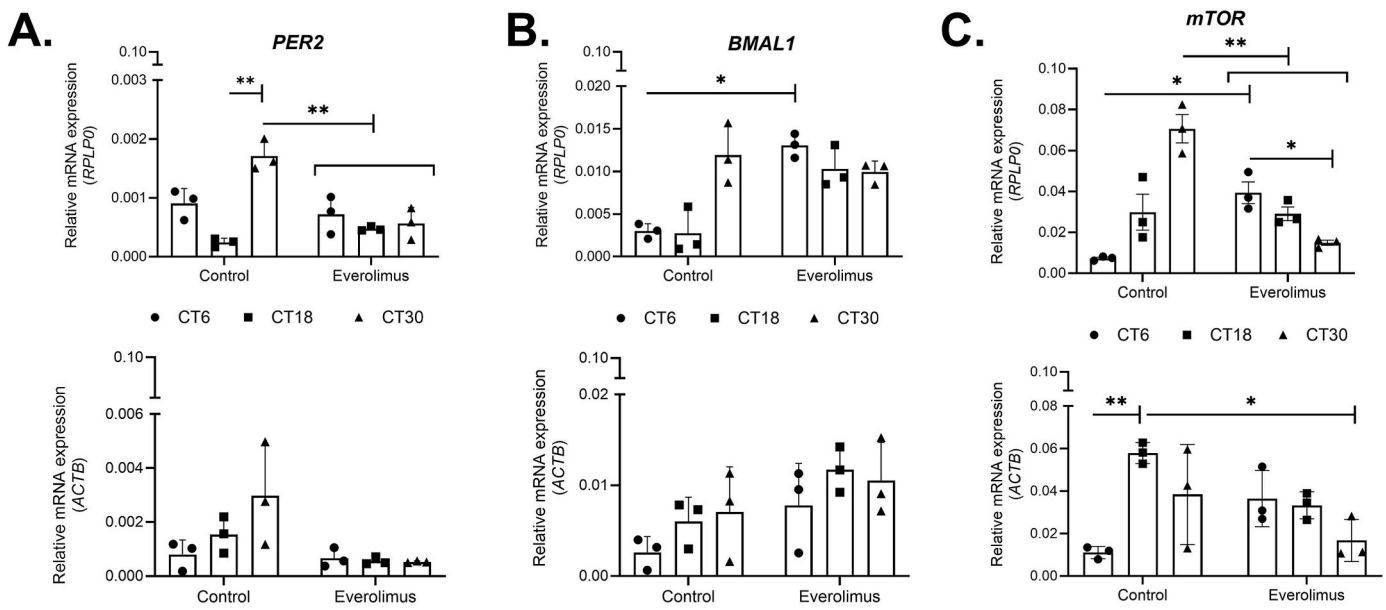

Fig. 6. Expression Patterns of PER2, BMAL1, and mTOR Following Everolimus Treatment. (A) PER2 expression significantly decreased at CT30 after everolimus exposure. (B) BMAL1 expression increased at CT6 when normalized to RPLP0. **(C)** mTOR expression showed time-dependent variation and was reduced most at CT30. *p < 0.05, **p < 0.01, ***p < 0.001.

acquisition, Conceptualization. **Sadullah Goncu:** Writing – review & editing, Methodology. **Alper Okyar:** Writing – review & editing, Funding acquisition.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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